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Layer-by-layer printing of cells and its application to tissue engineering

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ABSTRACT

Tissues and organs exhibit distinct shapes and functions nurtured by vascular connectivity. In order to mimic and examine these intricate structure-function relationships, it is necessary to develop efficient strategies for assembling tissue-like constructs. Many of the top-down fabrication techniques used to build microelectromechanical systems, including photolithography, are attractive due to the similar feature sizes, but are not suitable for delicate biological systems or aqueous environments. A layer-by layer approach has been proposed by us to pattern functional cell structures in three dimensions. Freeform cell structures are created by the inkjet method, in which cells are entrapped within hydrogels and crosslinked on demand. The cells are viable, functional and show potential for cell maturation as exemplified by the diversion of hematopoietic stem cells into multiple cell types. These results show promise for many tissue engineering applications.

INTRODUCTION

Tissue engineering may be defined as a multidisciplinary specialty applying the methods and principles of engineering and the life sciences toward the development of biological substitutes for the restoration, maintenance and improvement of organ function [1]. Traditionally this has been done by the seeding of cellular material onto a suitable scaffold material to create three dimensional (3D) constructs. [2] However there are currently a number of drawbacks to this technique. Organs consist of multiple cellular phenotypes in specific locations and this is hard to replicate. In addition, the degree of cellular permeation is variable and may not proceed uniformly. The volume of tissue that can be constructed is limited by diffusion and osmosis. Finally, though there have been large advances in scaffold technology the construction of contractile structures like the myocardium and vascular conduits continues to be a challenge. An alternative approach to tissue construction is a genuine challenge. Since the first application of fused deposition modeling for tissue engineering scaffolds [3], considerable effort has been focused on printing synthetic biodegradable scaffolds [4]. Concurrently a variety of rapid prototyping techniques have been developed to define macroscopically the shapes of

deposited biomaterials, including photolithography [5], syringe-based gel deposition [6], and solid freeform fabrication [7;8]. That these approaches have not yet led to the construction of harmonically organized complex tissues may be due to the difficulty to embed the various cell types within the intricate designs. Bottom up construction processes may offer a possible solution. Such processes have combined soft lithography and self-assembly to construct hierarchical non-living materials [9], and may be applied to the self-assembly of tissues or the study of cooperative effects in developmental biology [10]. Our approach combines rapid prototyping procedures with micro encapsulation to print viable freeform structures using custom modified printers [11-13]. We have previously shown that cell aggregates fuse when placed near each other on collagen hydro gel surfaces. This model system was chosen in an attempt to construct more complex three-dimensional tissue blocks by the combination of computer programmed deposition of cells or cell aggregates into macroscopically defined shapes. Moreover, this process known as computer-aided tissue engineering (CATE) [14] is based on the observation that cells have a biologically-programmed capacity for self-assembly and will differentiate during a short maturation process if provided a suitable environment, exemplified by the behavior of embryonic tissue explants [15]. We report here the fabrication of tubular cell structures that exhibit form and function.

MATERIALS AND METHODS

Preparation of Alginate
For fabricating the composite construct, sodium alginate (2% w/v) (Fisher Scientific, Pittsburgh, PA) and gelatin (0.1 w/v) was dissolved in Dulbecco's phosphate buffered saline (DPBS) (Sigma Chemicals, St. Louis, MO) overnight and the solution was filtered using 0.22 μm filter (Fisher Scientific, Pittsburgh, PA). As a cross linking solution, 0.25 M of $CaCl_2$ in double-distilled water was used. When $CaCl_2$ and sodium alginate are in contact, cross linking takes place forming a chemically reversible gel [16].

Thermal ink-jet printer
For this study we modified a HP697c inkjet printer to engineer freeform constructs. In brief, the paper feed mechanism of the printer was disabled and a z-axis module was incorporated. Since the z-axis control is very essential to build a 3-dimensional structure, an electronically controlled elevator chamber was designed and constructed using a stepper motor assembly. For the elevator stage a 2.5 cm round glass cover slip (Fisher Scientific, Pittsburgh, PA) was fixed to the tip of a metallic rod, which was connected to the stepper motor. A DC power supply (Hewlett-Packard Corp., Palo Alto, CA) was used to generate a 4V signal to operate the stepper motor through a series of four toggle switches. A sterile 50 ml conical tube (Fischer Scientific, Pittsburgh, PA) was customized and used as the elevator chamber and filled with of sterile alginate solution. A commercial inkjet cartridge (HP51629A, 40ml) was emptied and rinsed thoroughly with double distilled water and 100 % ethanol several times till completely free of ink. It was then immersed in an ultrasonic sonicator to remove any ink or blockages. The cartridge was sprayed with 100 % ethanol and the whole assembly of the printer along with the cartridge was placed overnight in a Class II hood under ultraviolet light prior to use.

Construction of cell tubes

Vital to cell patterning procedures is the use of stable, aqueous non-cytotoxic inks that act as cross linking agents delivered using the inkjet method into a rapid prototyping chamber. For this purpose we use ionic cross linking additives such as $CaCl_2$ in saline cell suspensions at hypertonic concentrations, thereby facilitating the passage of the cells through the printer nozzles as well as rapid local gelling of the pattern. Saline alginate solution was used in the chamber, ensuring biocompatibility of the structures. As a pattern of ink is printed onto the alginate meniscus, ion exchange causes cross linking of alginate, thereby facilitating cell encapsulation within the droplets ranging in diameter from 30 to 50 μm.

Alginate tube constructs of Rat smooth muscle cells (SMCs) were printed. The ink-jet cartridge was filled with 1.5 ml of 0.25 M $CaCl_2$ solution. Hybond paper (Amersham Pharmacia Biotech, Ireland) was placed on the cover slip of the z- stage and the tubes were printed on this paper. A tube pattern was designed in a MS Word document (Microsoft, Redmond, WA) using circles of 2 mm diameter and 2 mm width. After every print action a re-coating procedure was performed by lowering the elevator platform into the alginate filled chamber and brought back 100 microns below the original position utilizing the stepper motor assembly. This step was done to ensure the gelling of the printed rings. In order to maximize the amount of SMC cells used, 0.25 ml of the cell suspensions were manually pipetted onto the gelled rings after each re-coating procedure. The stacked rings gelled atop each other to create a cylindrical tube entrapping the cells. The tubes were carefully detached from the Hybond paper and individually placed in small polystyrene dishes (Fisher Scientific, Pittsburgh, PA) filled with 3 ml DMEM, which was changed every 24 hours. To test the viability of the encapsulated cells, a live dead assay (Molecular Probes, Eugene, OR) was done after 8 days of culture.

Confocal Microscopy

Cell distribution and morphology in the three dimensional constructs were examined under an LSM-5 spectral confocal microscope. The reconstruction of the top-down and side views and the color depth coding for the entrapped cells were accomplished using Zeiss LSM Image Examiner and Browser software. The SMC cell constructs were fixed on day 18 in 100 % ethanol for 1 hour. They were then rinsed well in DPBS. The cells were tagged with mouse monoclonal anti-actin, α-smooth muscle- FITC antibody (F3777-Fisher Scientific, PA) that was diluted in PBS at a ratio of 1:100 overnight at 4 °C. The constructs with the QCE6 cells were fixed on day 8 in 100 % ethanol for 1 hour. Since these cells express green fluorescent proteins, they were not tagged for viewing under the microscope. Both these samples were then cover slipped and stored in a cool dark place.

Functional testing of smooth muscle cell tubes

A 50nM solution of Endothelin-1 (ET-1, American peptide Company, Inc., Sunnyvale, CA) was prepared in serum free DMEM. The contractile response of smooth muscle cells encapsulated in the alginate tubes to ET-1 stimulation was evaluated over a period of 5 days at regular time intervals. The SMC tubes were incubated in 5 ml of DMEM supplemented with 10% of FBS in a culture dish at 37° C for 24h. The culture medium

was then replaced with serum free DMEM to arrest cell growth. After 24h of incubation in serum free medium, 50nM ET-1 solution was administered and after 48h, the tube was re-incubated in 5 ml of serum free media. The lumen area of the tube was measured daily up to 5 days by imaging the dishes with a digital scanner (Perfection 1640SU Photo, Epson) throughout the experiments. The lumen area was calculated using Scion Image software (Scion Corporation, Frederick, MD) and plotted against the time intervals for triplicate experiments.

RESULTS
Alginate tubes with encapsulated smooth muscle cells are shown in Figure 1 on days 0, 13 and 18. This figure depicts a distinct difference attributed to cellular activity. The smooth muscle cell phenotype was maintained and the cells were distributed evenly throughout the structure as judged by immunostaining confocal microscopy. Within the structure, the cells reached a density of 14×10^6 cells/ml with 80% viability, as determined by a vital stain at day 18 of the culture. Morphological analysis of histological slides corroborated these findings.

Although the morphological evidence for cell viability and distribution of cells in the tube is very compelling, development of vasomotor reactivity in the constructs should be a clear indication that the tubes are becoming a functional in vitro. In an attempt to show the function of the printed tubes, we studied the response of the tubes when challenged with the vasoconstricting agonist Endothelin-1 (ET-1). The tubes contracted in a dose-dependent manner upon exposure to the agonist with the responses shown in Figure 2, and complete closure of the lumen after 43 hrs in a 50 nM ET-1 solution. Although it is difficult to make a direct comparison with contractile responses of other tissue engineered vessels, the tubes clearly showed a functional response. In addition to this contractile response, a relaxation to the initial lumen diameter was seen after removal of the agonist. Clearly, both the challenge and the potential of organ printing technology lie in the design of functional tissues. These cell tubes exhibited vasoreactivity to agonists, while maintaining sufficient mechanical strength, demonstrating the potential utility of this technology to construct functional tissues from the bottom-up.

Tissue engineering has progressed in areas relevant to the developments of novel biomaterials [17], the designs of bioreactors for dynamic in vitro culture systems [18], and the uses of diverse cell sources including appropriate multi-potent stem cells [19]. For the construction of soft tissues or even entire organs, however, huge technical challenges remain to be overcome. Among them, the very important problem of how to combine and orchestrate cells, growth factors, and scaffolds into the tissue's architecture, especially where distinct cell types are required in anatomically exact locations to attain biological function. The inkjet printing technology offers a possible solution.

Figure 1. Macroscopic and microscopic images of printed cell tubes. (A) Constructs with SMC cells as seen on day 0 immediately after printing (B) a SMC cell tube incubated in media on day 13, (C) Cell tubes on day 8 (D) vital stain of SMC cells on day, (E) 1 vital stain of SMC cells on day 18, and (F) confocal image of SMC cell tubes tagged with FTIC labeled monoclonal anti-actin, α-smooth muscle antibody on day 18.
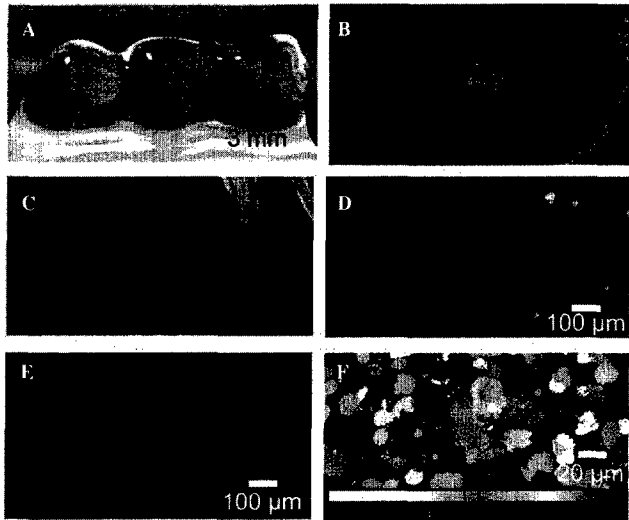

Figure 2. Effect 50 nM of ET-1 on of lumen size of a short SMC tube. (A) Construct after 24 h in DMEM augmented with 10% FBS, (B) Construct after 12 h incubation in serum free medium augmented with 50 nM of ET-1, (C) Construct after 24 h in the ET-1 containing medium, (D) Construct after 45 h in the ET-1 containing medium, (E) Construct after an additional 12 h in the FBS containing medium, (F) Construct after 24 h in the FBS medium.
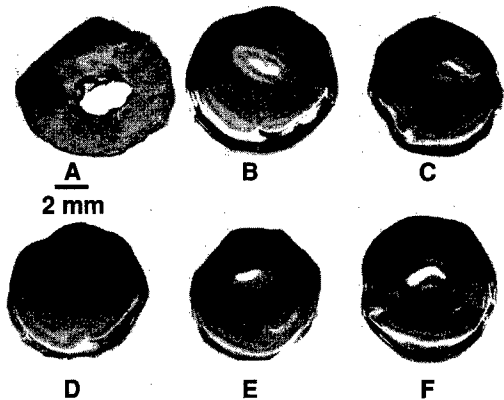
CONCLUSIONS

To our knowledge no other group has sought to use a commercial inkjet printer as a tool to print alternate layers of hydrogels and cells. We have previously shown that mammalian cells can be printed using inkjet printers [20]. The results presented here show promise in constructing more complex three dimensional shapes. This method, when combined with cell printing offers the capability of arranging viable cells directly into specific patterns, which is critical for building engineered tissue or in vitro models to study cooperative effects in systems biology. We conclude that a combined approach using freeform fabrication techniques aided by the self-assembly nature of cells may be a viable approach to construct building blocks for soft tissues.

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